

A purine-rich intronic element enhances alternative splicing of thyroid hormone receptor mRNA

MICHELLE L. HASTINGS,^{1,2} CATHERINE M. WILSON,^{1,3} and STEPHEN H. MUNROE¹

¹Department of Biology, Marquette University, Milwaukee, Wisconsin 53201, USA

ABSTRACT

The mammalian thyroid hormone receptor gene *c-erbA α* gives rise to two mRNAs that code for distinct isoforms, TR α 1 and TR α 2, with antagonistic functions. Alternative processing of these mRNAs involves the mutually exclusive use of a TR α 1-specific polyadenylation site or TR α 2-specific 5' splice site. A previous investigation of TR α minigene expression defined a critical role for the TR α 2 5' splice site in directing alternative processing. Mutational analysis reported here shows that purine residues within a highly conserved intronic element, SE α 2, enhance splicing of TR α 2 *in vitro* as well as *in vivo*. Although SE α 2 is located within the intron of TR α 2 mRNA, it activates splicing of a heterologous *dsx* pre-mRNA when located in the downstream exon. Competition with wild-type and mutant RNAs indicates that SE α 2 functions by binding *trans*-acting factors in HeLa nuclear extract. Protein–RNA crosslinking identifies several proteins, including SF2/ASF and hnRNP H, that bind specifically to SE α 2. SE α 2 also includes an element resembling a 5' splice site consensus sequence that is critical for splicing enhancer activity. Mutations within this pseudo-5' splice site sequence have a dramatic effect on splicing and protein binding. Thus SE α 2 and its associated factors are required for splicing of TR α 2 pre-mRNA.

Keywords: alternative splicing; hnRNP F; hnRNP H; RNA-binding proteins; SF2/ASF; splicing enhancer

INTRODUCTION

Alternative mRNA processing is an important mechanism for generating multiple mRNAs and proteins from a single gene (reviewed in Smith & Valcárcel, 2000). In mammals, transcripts from the *c-erbA α* gene (NR1A1; Nuclear Receptors Committee, 1999) code for two major thyroid hormone receptor (TR) isoforms, TR α 1 (NR1A1a) and TR α 2 (NR1A1b), which are functionally antagonistic (for a review, see Lazar, 1993). TR α 1 is an authentic thyroid hormone receptor that binds thyroid hormone (T3) and mediates its activity in many cells and tissues. TR α 2 is a variant receptor that lacks a functional hormone-binding site and antagonizes the ability of TR α 1 to activate gene transcription in the presence of T3 (Koenig et al., 1989; Lazar et al., 1989a). Although both isoforms are expressed in most mammalian cells, the levels of each

vary widely in different tissues and at different stages of development. Thus, regulation of TR α alternative processing is important for determining cellular levels of TR α 1 and TR α 2 mRNAs, which, in turn, are critical for modulating the response to T3.

TR α 1 and TR α 2 mRNAs are distinguished by their distinct 3' terminal exons. To produce TR α 1 mRNA, TR α pre-mRNA is cleaved and polyadenylated at the penultimate exon (exon 9; Lazar et al., 1989b; Laudet et al., 1991). Alternatively, TR α 2 mRNA results from use of a 5' splice site within exon 9 and ligation to an exon (exon 10) directly downstream of the TR α 1-specific poly(A) site. Thus, TR α 2 lacks most of the TR α 1 terminal exon but includes an alternative 3' exon.

Alternatively processed exons often are characterized by suboptimal splice signals that are recognized inefficiently in the absence of auxiliary sequences or *trans*-acting factors (for a review, see Berget, 1995; Smith & Valcárcel, 2000). In the case of TR α 2 mRNA splicing, a single nucleotide deviation from the 5' splice site consensus sequence is responsible for weak splicing activity. Mutation of the nonconsensus +5C residue in the natural TR α 2 5' splice site to a consensus +5G results in constitutive processing of TR α 2 mRNA with complete exclusion of the TR α 1 mRNA processing (Hastings et al.,

Reprint requests to: Stephen H. Munroe, Department of Biology, Wehr Life Sciences Building, Marquette University, P.O. Box 1881, Milwaukee, Wisconsin 53201-1881, USA; e-mail: stephen.munroe@marquette.edu.

²Present address: Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724, USA.

³Present address: Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226, USA.

2000). Efficient processing of such suboptimal splice sites often requires *cis*-acting sequence elements, referred to as splicing enhancers, that facilitate exon recognition through interactions with the splicing machinery (for a review, see Hertel et al., 1997). Many splicing enhancers identified to date are purine-rich sequences located within downstream exons. These enhancers interact with members of the serine-arginine (SR)-rich family of splicing proteins and promote the use of weak 3' splice sites in the upstream intron (for a review, see Graveley, 2000). The ability of individual SR proteins to recognize specific enhancer sequences and influence splicing in a dose-dependent manner may be an important mechanism for regulating alternative processing.

In addition to the exonic, purine-rich elements described above, a number of splicing enhancers are found within introns (Gooding et al., 1994; Huh & Hynes, 1994; Del Gatto & Breathnach, 1995; Gallego et al., 1997; Wei et al., 1997; Carstens et al., 1998; Kosaki et al., 1998; Lim & Sharp, 1998; Lou et al., 1998; McCarthy & Phillips, 1998; Chou et al., 1999). The sequences of intronic splicing enhancers identified thus far are diverse and often distinct from the purine-rich splicing enhancers. Consistent with the differences in sequence, proteins bound to intronic splicing enhancers are usually different from the SR proteins that bind purine-rich enhancers. The activity of splicing enhancers may be regulated by the nature and amount of specific RNA-binding proteins within the cell nucleus. Other variables are also likely to be important, including the locations of multiple enhancer and repressor elements, and the strength and position of adjacent or competing processing sites. Characterization of such regulatory elements is important for understanding the mechanisms regulating alternative pre-mRNA processing.

In this study we characterize a splicing enhancer located within the final intron of TR α 2 mRNA, 130 nt downstream of the TR α 2-specific 5' splice site. We show that this highly conserved enhancer sequence stimulates TR α 2 mRNA splicing *in vivo* and *in vitro* and interacts with *trans*-acting factors that include two abundant, specifically bound proteins of 34 and 55 kDa corresponding to SF2/ASF and hnRNP H. We also identify a pseudo-5' splice site sequence within the splicing enhancer element that is important for enhancer activity. Our results support a model in which the intronic element helps determine the relative levels of TR α 1 and TR α 2 mRNAs by enhancing the activity of the weak TR α 2-specific 5' splice site upstream.

RESULTS

Mutations in SE α 2 inactivate splicing enhancer activity

Previous analysis demonstrated that a highly conserved sequence immediately downstream of the TR α 1

coding sequence promotes TR α 2-specific splicing (Hastings et al., 2000). These studies used a deletion mutant, pErbA(Δ BS), that lacks all but 300 nt of the 3.7-kb TR α 2-specific intron (Fig. 1A) and is severely deficient in TR α 2-specific splicing (Hastings et al., 2000). Addition of 80 nt at the 5' end of the Δ BS deletion in pErbA(SE80) restores efficient splicing (Fig. 1B; Hastings et al., 2000). The sequence of this 80-nt splicing enhancer element, SE α 2, is very purine rich, containing 73% A and G (Fig. 1A). SE α 2 contains three long uninterrupted stretches of purines (underlined in Fig. 1A) that resemble the GAR (R = purine) repeats present in many previously characterized exonic splicing enhancers (Xu et al., 1993; Tanaka et al., 1994; Humphrey et al., 1995; reviewed in Hertel et al., 1997).

To determine whether the purine-rich composition of SE α 2 or, more specifically, the GAR repeats within this region, play a role in its activity, the sequence of SE80 was disrupted by introducing two different types of mutations. First, six A-to-G and two G-to-A substitutions were made in pErbA(SE80), replacing the alternating G/A sequence of the GAR-like repeats in SE80 with runs of three to five consecutive A or G residues (G/A, Fig. 1A). These substitutions resulted in a twofold decrease in splicing relative to the pErbA(SE80) transcript (Fig. 1B, lanes 5 and 6; Fig. 1C). Thus, a relatively limited change, involving only eight purine residues near the center of an intron nearly 300 nt long, causes a substantial change in TR α 2 splicing. This result demonstrates that the GAR repeats are important for the function of this splicing enhancer.

An even more dramatic effect on splicing was observed when G residues located throughout the 80-nt region were changed to U residues (G/U, Fig. 1A). Only 7.5% of the mRNA was spliced in transcripts containing the SE80G/U mutation, an 80% decrease relative to wild-type SE80 (Fig. 1B, lanes 7 and 8; Fig. 1C). Because this level of splicing is nearly identical to that of Δ BS (Fig. 1B, lanes 9 and 10), which lacks SE α 2, these G/U substitutions completely inactivate the enhancer element.

SE α 2 is required for efficient TR α 2 splicing *in vitro*

Next, the activity of SE α 2 was examined in a system optimized for TR α 2 splicing *in vitro* (Munroe & Lazar, 1991). To this end, a series of truncated TR α transcripts were prepared that contain the 5' portion of exon 9, including the TR α 2-specific 5' splice site, along with regions from both the 5' and 3' ends of the TR α 2 intron, and 250 nt of exon 10. Transcripts containing intron deletions identical to those of pErbA(Δ BS), pErbA(SE80), and pErbA(SE80)G/U minigenes were assayed for splicing in HeLa cell nuclear extracts (Fig. 2A). The relative splicing levels of the three precursor RNAs were comparable to those observed

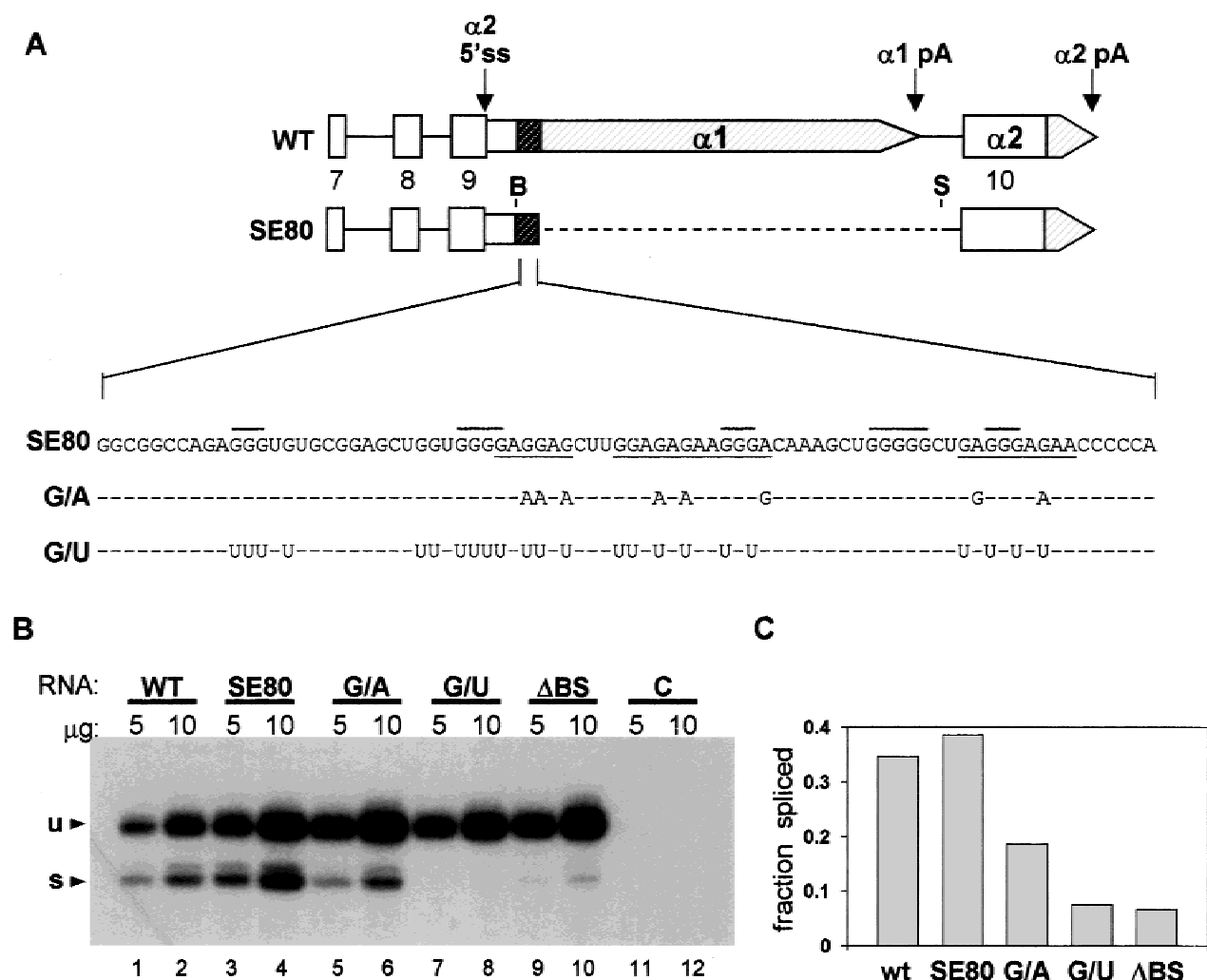


FIGURE 1. Mutations in SE α 2 inhibit TR α 2 mRNA splicing. **A:** Structure of the wild-type (WT) and SE80 minigene. Arrows show positions of the TR α 2-specific 5' splice site and polyadenylation (pA) sites for TR α 1 and TR α 2 and the dark box represents the SE α 2 splicing enhancer. Vertical lines show restriction sites for *Bsu*36I (B) and *Ssp*I (S) that define the Δ BS deletion. Exons (boxes) are numbered from the 5' end of the *c-erbA α* gene. Exons in TR α 2 mRNA are thicker than the region of exon 9 unique to TR α 1. Open boxes indicate coding regions, and hatched boxes untranslated regions. The sequence of the wild-type and mutant SE80 splicing enhancer is shown below. Purine-rich GAR-like sequences are underlined and G tracts are overlined. **B:** RNase protection assays of total RNA from COS-1 cells transfected with the indicated pErbA plasmids using a probe complementary to the TR α 2-specific 5' splice site. As a control, the probe was hybridized with tRNA (C, lanes 11 and 12). Spliced (s) and unspliced (u) RNA is indicated at left. **C:** Quantitation of splicing as a fraction of spliced TR α transcript. Histogram represents the average of at least two experiments.

in vivo (Fig. 2B). About three times as much of the p α 2(SE80) transcripts were spliced relative to p α 2(Δ BS) and p α 2(SE80G/U) transcripts after 2.5 h. These results demonstrate that SE α 2 functions as a splicing enhancer in vitro.

SE α 2 enhances splicing of drosophila *dsx* chimeric pre-mRNA

Analysis of SE α 2 in vitro provides an approach for examining SE α 2 enhancer activity in the context of a heterologous pre-mRNA. Several exonic purine-rich enhancers that closely resemble the sequence of SE α 2

have been shown to promote splicing of exons 3 and 4 of a truncated *Drosophila dsx* (Fig. 3A), that is spliced very poorly in the absence of its own enhancer elements (Tian & Maniatis, 1993). To determine if SE α 2 is functionally similar to other purine-rich enhancers, we tested mutations and length variants of SE α 2 fused to the 3' end of the truncated exon 4 (Fig. 3A).

First, a *dsx* chimeric transcript containing SE80 (*dsx*SE80) was assayed for *dsx* splicing activity (Fig. 3B,C). As controls, an enhancerless *dsx* transcript (*dsx*) and a transcript that includes the strong purine-rich splicing enhancer element from avian sar-

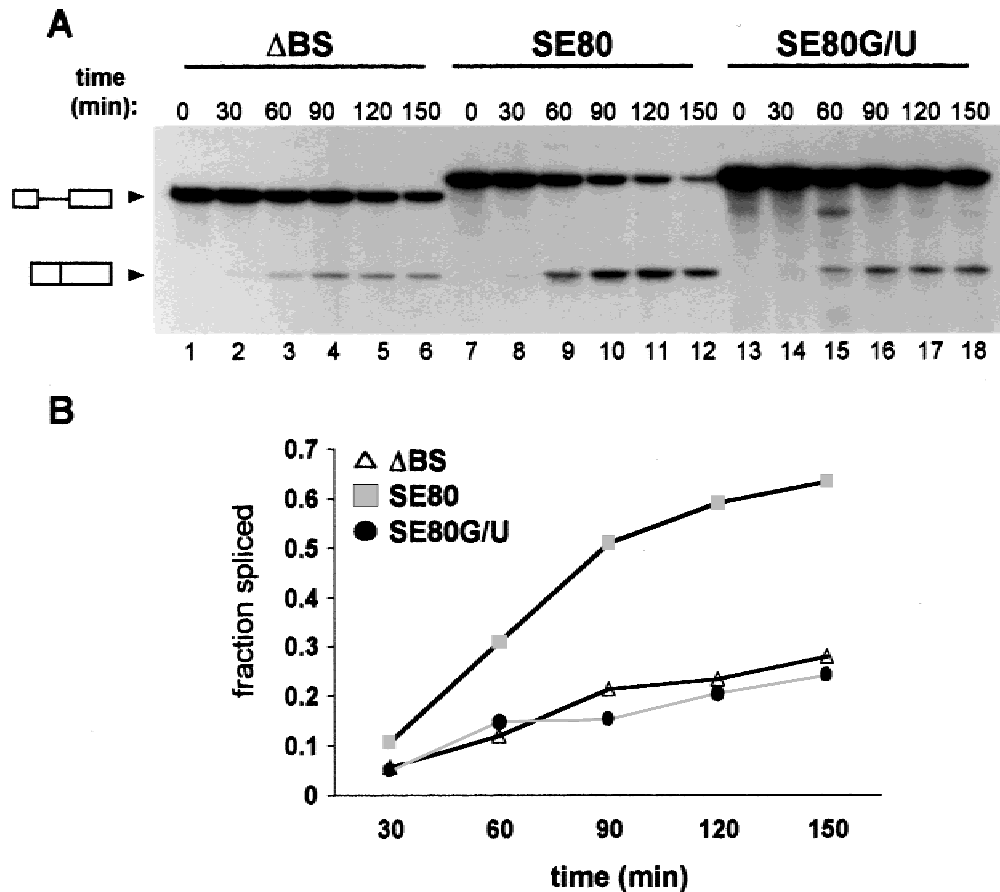


FIGURE 2. The splicing enhancer SE α 2 stimulates TR α 2 splicing in vitro. **A:** In vitro splicing assays carried out in HeLa nuclear extract. Reaction times are indicated above the lanes. Schematics of unspliced and spliced RNAs are shown at the left of the panel. **B:** The graph shows the time course of splicing, expressed as the fraction of spliced RNA. The numbers are an average of 2–4 experiments with the exception of the 90- and 150-min time points, which represent one experiment.

coma leukemia virus (dsxASLV; Staknis & Reed, 1994) were tested in parallel. As expected, dsx pre-mRNA without a splicing enhancer was processed inefficiently (Fig. 3B, lane 2; Fig. 3C). In contrast, the presence of the ASLV enhancer resulted in an eightfold stimulation of dsx splicing (Fig. 3B, lane 4). Under identical conditions, the addition of SE80 to dsx enhanced splicing more than threefold (Fig. 3B, lane 6). These results show that SE80 functions as a splicing enhancer in a heterologous pre-mRNA.

To determine if sequences between the TR α 2 5' splice site and SE80 also contribute to enhancer activity, a fragment containing an additional 80 nt upstream of SE80 (dsx80 + SE80) was also tested. This transcript was spliced at nearly equivalent levels as dsxSE80 (Fig. 3B, lane 10, 3C). This result suggests that sequences upstream of SE80 do not contribute to activity of SE α 2, as assayed in the context of dsx. The specificity of SE α 2 enhancer activity was tested using the SE80G/U mutation (dsxSE80G/U; Fig. 3B, lanes 7 and 8) and SE80(A/G) mutation (not

shown) in the context of the dsx pre-mRNA. Replacement of SE80 with SE80G/U in the dsx chimeric transcript resulted in a nearly eightfold decrease in splicing (Fig. 3C). This result is consistent with the lack of enhancer activity observed both in vivo and in vitro with TR α pre-mRNAs containing this mutation. However, the dsxSE80A/G mutation showed identical levels to wild-type RNA (not shown), suggesting that in vitro enhancement of splicing in the context of the heterologous dsx exon does not require the GAR repeats of the wild-type SE80 sequence.

Trans-acting factors are required for SE α 2 activity

The activity of SE α 2 in vitro suggests that *trans*-acting factors present in the nuclear extract mediate enhancer activity. To determine whether specific interactions with *trans*-acting factors are required for SE α 2 activity, competition experiments were performed. If SE α 2 binds factors required for TR α 2 splicing, an excess of SE α 2

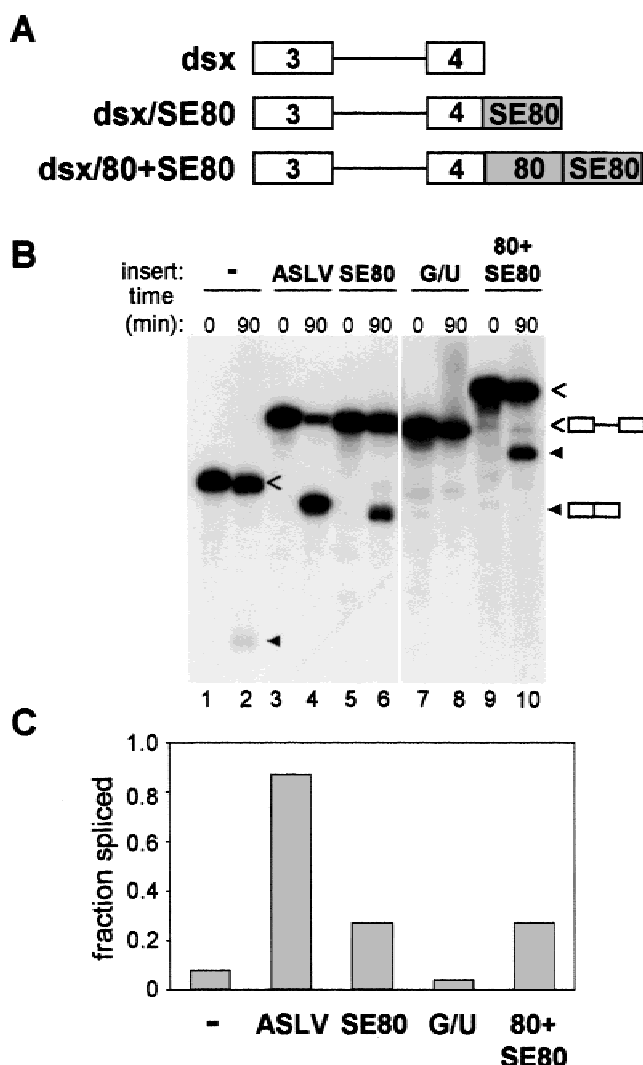


FIGURE 3. SE α 2 stimulates dsx pre-mRNA splicing. **A:** Diagram of the dsxSE80 chimeric transcripts. SE80, SE80 with G/U mutations (as in Fig. 1), and an extended SE α 2 with additional 5' sequences (80+SE80) were inserted downstream of the dsx minigene. **B:** In vitro splicing assays carried out for 90 min in HeLa nuclear extract. The dsx without an insert (–, lanes 1 and 2) and the dsx with a strong splicing enhancer (ASLV, lanes 3 and 4) were also included. Reaction products are shown schematically at right. Open arrowheads indicate unspliced RNA and black arrowheads show spliced products. **C:** Graphical representation of splicing activity.

RNA should competitively inhibit splicing. Splicing reactions containing 32 P-labeled p α 2(SE80) transcript were incubated with one of three short competitor RNAs, including wild-type and mutant forms of SE80 and an unrelated nonspecific sequence (NS) of the same length (Fig. 4A). Addition of 25–50 nM of the wild-type SE80 competitor RNA resulted in a 30–60% decrease in splicing (Fig. 4A, lanes 3–5). This decrease reflects specific competition by SE80 because addition of either the mutant competitor, SE80G/U (Fig. 4A, lanes 6–8) or the nonspecific competitor RNA (Fig. 4A, lanes 9–11) had a smaller effect on splicing than SE80. Even at the

highest concentration tested, with competitor present at a 500-fold excess over the pre-mRNA, the wild-type competitor inhibited splicing more than either the mutant or nonspecific RNAs (Fig. 4B), demonstrating the specificity of competition by SE80. This result indicates that *trans*-acting factors in HeLa cell nuclear extracts are required for SE α 2 activity.

To determine whether the factors mediating enhancer activity in these assays were specific for TR α 2 mRNA splicing, parallel experiments were also carried out with a constitutively spliced β -globin transcript (Krainer et al., 1984). Results of these experiments (Fig. 4A, lanes 12–22) indicate that SE80 and the two control RNAs inhibit splicing of β -globin pre-mRNA at levels very similar to that of the TR α pre-mRNA. Thus wild-type SE80 competitor RNA specifically sequesters factors that are required for constitutive as well as alternative splicing.

Proteins crosslink specifically to SE α 2 sequences

The next step was to characterize protein factors that mediate SE α 2 activity by binding specifically to SE80 RNA. Proteins bound to radiolabeled SE80 transcripts were crosslinked by UV irradiation following incubation in HeLa cell nuclear extract under splicing conditions. After trimming with ribonuclease and separating by SDS-PAGE, two prominent bands were observed, representing proteins of approximately 34 kDa and 55 kDa molecular weight (Fig. 5A,B, lanes 1). Several fainter bands, including one migrating at 22 kDa, were also observed. The specificity of protein crosslinking to SE α 2 was tested by pre-incubating nuclear extract with increasing amounts of unlabeled SE80 competitor and SE80G/U competitor (Fig. 5A). Under these conditions, binding of the 55-kDa and 34-kDa proteins was efficiently competed out by an excess of wild-type SE80 (Fig. 5A, lanes 2–4), but not with SE80G/U (Fig. 5A, lanes 5–7). Crosslinking of the 22-kDa protein was competed out by both specific and nonspecific competitors, as were the other fainter bands. Interestingly, the 34-kDa and 22-kDa proteins were crosslinked in both nuclear extract and SR protein containing reactions (Fig. 5B, lanes 1–3), but not in S100 extract (Fig. 5B, lane 6). Because SR proteins are depleted in S100 extract (Krainer & Maniatis, 1985), this result suggests that the 22-kDa and 34-kDa proteins represent SR proteins. SF2/ASF is one possible candidate for the 34-kDa protein because it is abundant in the SR fraction, migrates at the expected position and is efficiently crosslinked to SE80 (Fig. 5B, lanes 4 and 5; cf. Fig. 6A). In contrast, crosslinking of the 55-kDa band was observed in the nuclear and S100 extracts, but not in the SR protein fraction (Fig. 5B, cf. lanes 1 and 6 to 2 and 3). Therefore, the 55-kDa protein does not appear to be an SR protein.

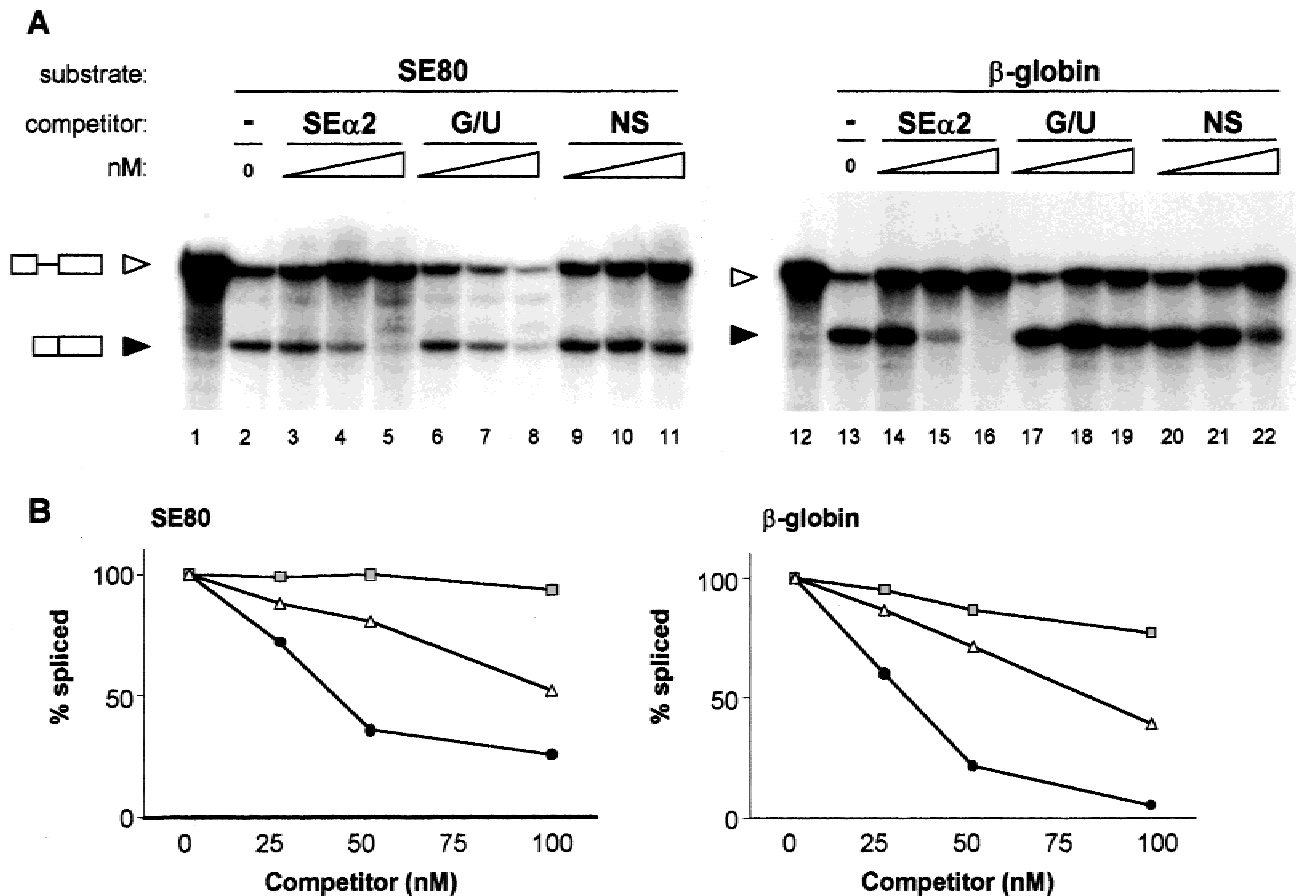


FIGURE 4. Inhibition of TR α 2 mRNA splicing in vitro by competition with SE80 RNA. **A:** HeLa nuclear extract was preincubated without competitor RNA (lanes 2 and 13) or with 25, 50, or 100 nM of the unlabeled competitor SE80 (lanes 3–5 and 14–16), SE80G/U (lanes 6–8 and 17–19), or a nonspecific RNA (NS, lanes 9–11 and 20–22). After preincubation, either α 2-SE80 pre-mRNA (lanes 1–11) or β -globin pre-mRNA (lanes 12–22) was added at a concentration of 0.2 nM and the incubation was continued for an additional 90 min, except for two reactions collected immediately (lanes 1 and 12) to show unspliced substrate. Open arrows indicate precursor RNA and black arrows show spliced products. **B:** Percentage of splicing, normalized to 100% in the absence of competitor RNA, is shown as a function of competitor concentration. Left panel shows splicing of α 2SE80 pre-mRNA and right panel splicing of β -globin pre-mRNA. Competitor RNAs with long flanking sequence were used and are indicated as circles: SE80; squares: SE80G/U; and triangles: nonspecific RNA.

SF2/ASF and hnRNP H bind specifically to SE α 2

As a second approach to identifying proteins that interact with SE α 2, northwestern blotting was performed. The radiolabeled SE80 probe bound primarily to the 34-kDa band in the SR protein fraction (Fig. 6A, top panel, lane 3). Western blotting carried out in parallel with northwestern blots showed that the 34-kDa band, the most intense band in the SR fraction, comigrated with SF2/ASF (Fig. 6A, bottom panel, lane 3). Recombinant SF2/ASF, which migrates substantially faster than endogenous SF2/ASF due to its lack of phosphorylation (Krainer et al., 1991), also bound labeled SE80 probe (Fig. 6A, bottom panel, lane 4). A faint band comigrating with SF2/ASF was also visible in the nuclear extract (Fig. 6A, bottom panel, lane 1). Additional northwestern blots demonstrated that labeled SE80(G/U)

did not bind SF2/ASF or proteins in the SR fraction (not shown). Also, recombinant SC35, another SR protein that comigrates with SF2/ASF, failed to bind SE α 2 under conditions where SF2/ASF bound efficiently (Fig. 6A, bottom panel, lane 5). The relative intensities of the bands in nuclear extract and SR fraction reflect the substantially higher concentration of SR proteins in the latter, as can be seen by comparing the corresponding lanes in the northwestern and western blots (Fig. 6A, lanes 1 and 3). The labeled SE80 probe also bound to a 90-kDa protein present in nuclear extract but not SR protein fraction (Fig. 6A, top panel, lanes 1–3). The 55-kDa protein that is efficiently crosslinked to SE80 apparently binds SE80 inefficiently under blotting conditions. However, a faint band in the 55-kDa region was detected upon longer exposures (not shown).

Immunoprecipitation experiments were carried out to confirm the binding of SF2/ASF to SE80 and to identify

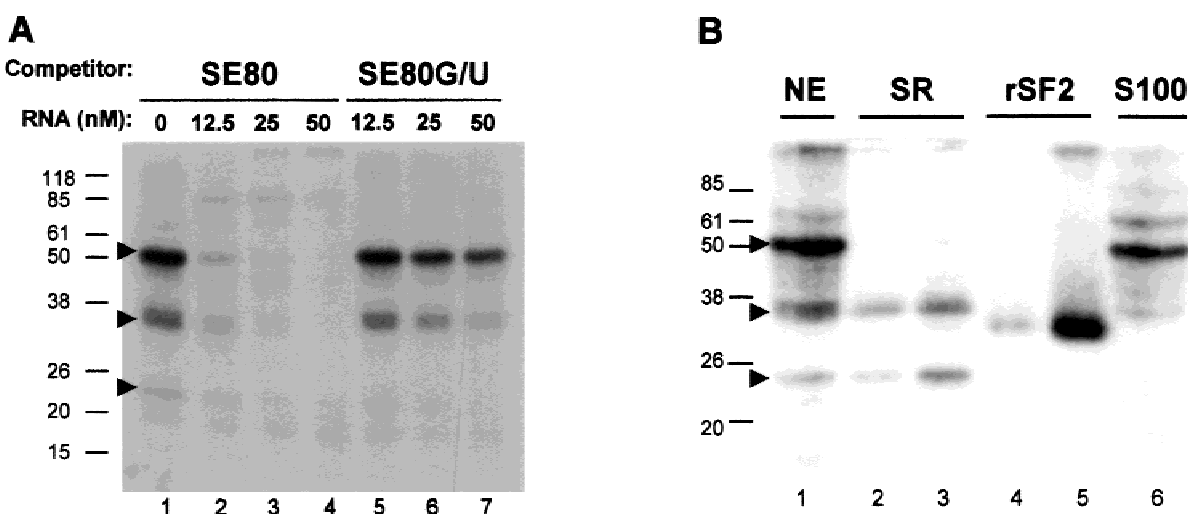


FIGURE 5. Nuclear proteins crosslinking to SE80. **A:** Protein crosslinking to 32 P-labeled SE80 RNA in HeLa cell nuclear extract with or without (lane 1) an excess of wild-type (lanes 2–4) or G/U mutant (lanes 5–7) unlabeled SE80 competitor RNA with short flanking sequence. Molecular weight markers are shown at the left of the panel. **B:** Crosslinking of proteins in nuclear extract (lane 1), HeLa SR proteins (lane 2, 150 ng; lane 3, 300 ng), recombinant SF2/ASF (rSF2; lane 4, 60 ng; lane 5, 120 ng), and S100 extract (lane 6) to labeled SE80 RNA with long flanking sequence. Arrowheads in **A** and **B** indicate three abundant proteins of 22, 34, and 55 kDa.

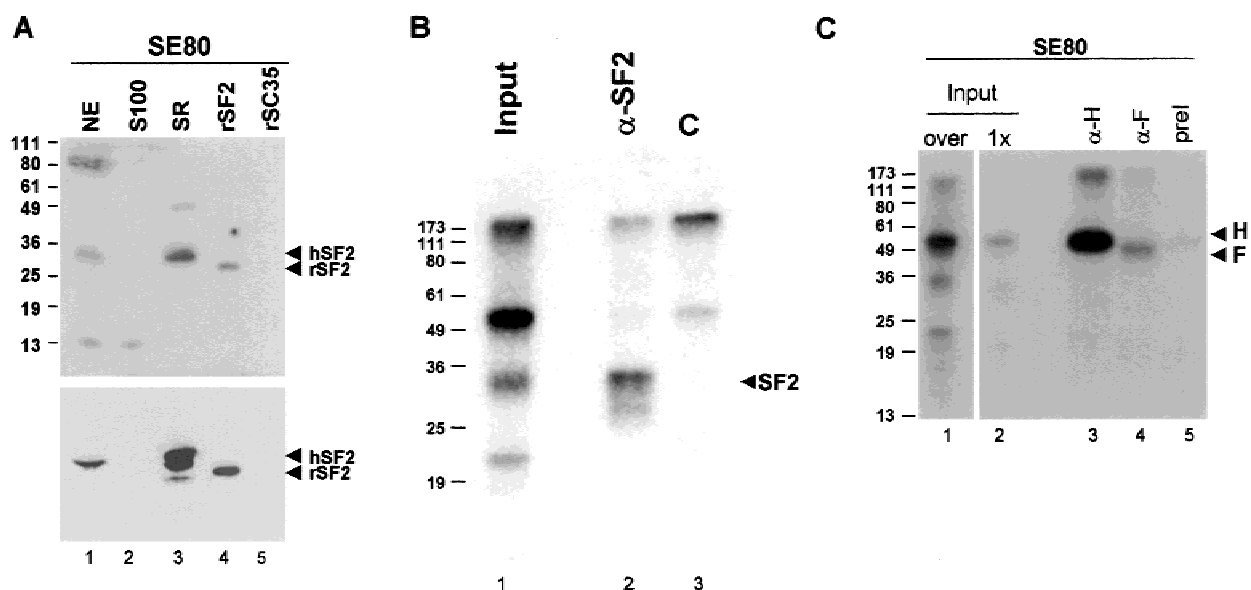


FIGURE 6. Binding of SF2/ASF, hnRNP H, and hnRNP F to SE80. **A:** Analysis of proteins binding to SE80. Upper panel: Northwestern blot of proteins binding SE80. Samples of nuclear extract (NE, lane 1), S100 extract (lane 2), HeLa SR proteins (lane 3, 0.6 μ g), recombinant SF2/ASF (lane 4, 0.1 μ g), and SC35 (lane 5, 0.1 μ g) were analyzed by SDS-PAGE, blotted, and probed with 32 P-labeled SE80 RNA. Lower panel: Parallel western blot analysis of gel run with the same samples. The blot was developed with SF2/ASF-specific antibody. The migration of endogenous, phosphorylated SF2/ASF (hSF2) from HeLa cells and recombinant, nonphosphorylated SF2/ASF are indicated at the right on both panels. **B:** Immunoprecipitation of crosslinked proteins with SF2/ASF-specific antibody. SE80 was incubated in nuclear extract under splicing conditions for 30 min and then UV crosslinked. Input lane (lane 1) represents 8% of the UV-crosslinked splicing reaction, which was subsequently immunoprecipitated with an SF2/ASF-specific antibody (lane 2) or a control antibody (lane 3). Proteins were resolved by SDS-PAGE on a 12% gel. **C:** Immunoprecipitation of labeled SE80 with hnRNP H- and F-specific antibodies. Input lanes at two exposures (lanes 1 and 2) are 4% of the reaction that was used in the immunoprecipitations using polyclonal antibodies specific for hnRNP H (lane 3), or hnRNP F (lane 4), or preimmune serum (lane 5).

the 55-kDa crosslinked protein. As shown in Figure 6B, SF2/ASF, labeled by UV crosslinking to SE80, was specifically precipitated with a monoclonal antibody specific for SF2/ASF (lane 2), but not by antibodies prepared in a similar fashion to a control antigen (lane 3). A similar approach was used to identify the abundant 55-kDa protein. Because this protein is present in the S100 extract but not the SR protein preparation, possible candidates include hnRNP F or hnRNP H, two closely related RNA-binding proteins that are known to bind to splicing regulatory elements (Chen et al., 1999; Chou et al., 1999; Fogel & McNally, 2000). As shown in Figure 6C, a polyclonal antibody specific for hnRNP H (Chou et al., 1999) efficiently precipitated a 55-kDa crosslinked protein comigrating with the most intense input band, thereby confirming that hnRNP H was cross-linked to SE80 (Fig. 6B, lane 3). A polyclonal antibody that specifically recognizes hnRNP F precipitated a small amount of crosslinked hnRNP F that migrates immediately below hnRNP H (Fig. 6B, lane 4). On the basis of these results, we conclude that SF2/ASF and hnRNP H are two of the major proteins that bind to SE α 2.

A 5' splice site-like sequence is required for SE α 2 activity

To further analyze the role of specific sequences and proteins required for the function of SE α 2, a series of deletion mutations was constructed, transfected into HEK 293 cells, and analyzed by RNase protection

assays using the TR α 2 5' splice site-specific probe (Fig. 7A). SE70, SE60, SE50, and SE40 contain deletions of 10–39 nt from the 5' end of SE80. SE80 Δ 30 contains a deletion of 30 nt spanning the coding sequence for the last 10 amino acids of TR α 1, immediately upstream of the 5' end of SE80 (Fig. 7A). SE60/3' contains a deletion of 20 nt from the 3' end of the SE80 enhancer. The splicing of SE80 Δ 30 and SE70, containing deletions upstream and at the 5' end of SE80, respectively, is about 90% that of SE80, demonstrating that sequences more than 10 nt downstream of the Bsu36I site account for most of the enhancer activity (Fig. 7B, lanes 1–3). A 20-nt deletion from either end of the enhancer fragment in SE60 and SE60/3' had a significant effect on splicing, with a 25% and 33% decrease, respectively, relative to SE80 (Fig. 7B, lanes 4 and 7). Deletions of 30–40 nt from the 5' end of SE80 had a much more drastic effect on splicing as seen with SE50 and SE40, which are spliced at a level only 20–40% that of the full-length SE80 (Fig. 7B, lanes 5 and 6). Previous results further showed that deletion of 50 nt from the 5' end of SE80 eliminates splicing enhancer activity (Hastings et al., 2000). The substantial but partial loss of enhancer activity seen with the deletion of 20 nt from either end of the enhancer (Fig. 7, lanes 1, 4, and 7) and with the SE80G/A substitutions (Fig. 1C) strongly suggests that the SE α 2 splicing enhancer comprises multiple elements that interact to promote splicing of TR α 2 mRNA.

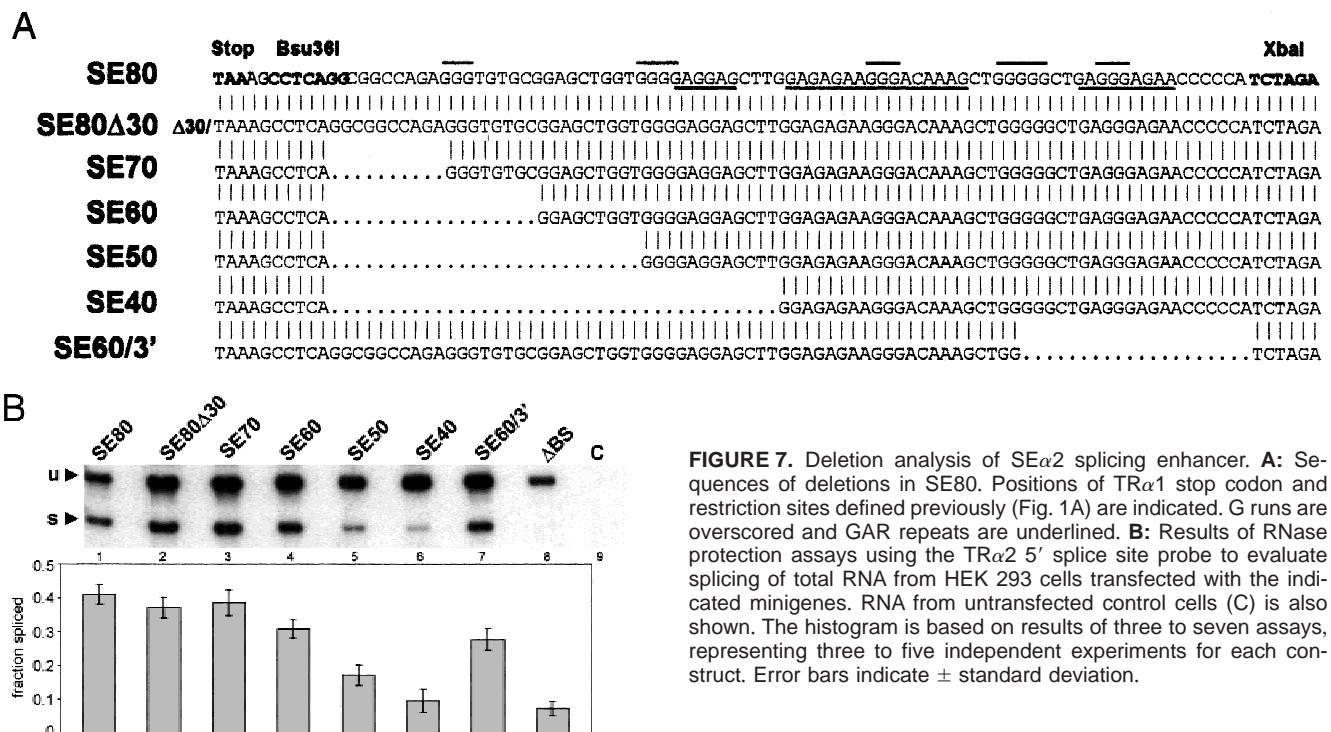


FIGURE 7. Deletion analysis of SE α 2 splicing enhancer. **A:** Sequences of deletions in SE80. Positions of TR α 1 stop codon and restriction sites defined previously (Fig. 1A) are indicated. G runs are overscored and GAR repeats are underlined. **B:** Results of RNase protection assays using the TR α 2 5' splice site probe to evaluate splicing of total RNA from HEK 293 cells transfected with the indicated minigenes. RNA from untransfected control cells (C) is also shown. The histogram is based on results of three to seven assays, representing three to five independent experiments for each construct. Error bars indicate \pm standard deviation.

Further analysis focused on the dramatic drop in splicing enhancer activity between SE60 and SE50, which differ by only 9 nt (Fig. 7A,B, lanes 4 and 5). This region

at the 5' end of SE60 includes the sequence CTG GTGGGG (Fig. 8A, top), which closely resembles the mammalian 5' splice site consensus sequence (CAG/

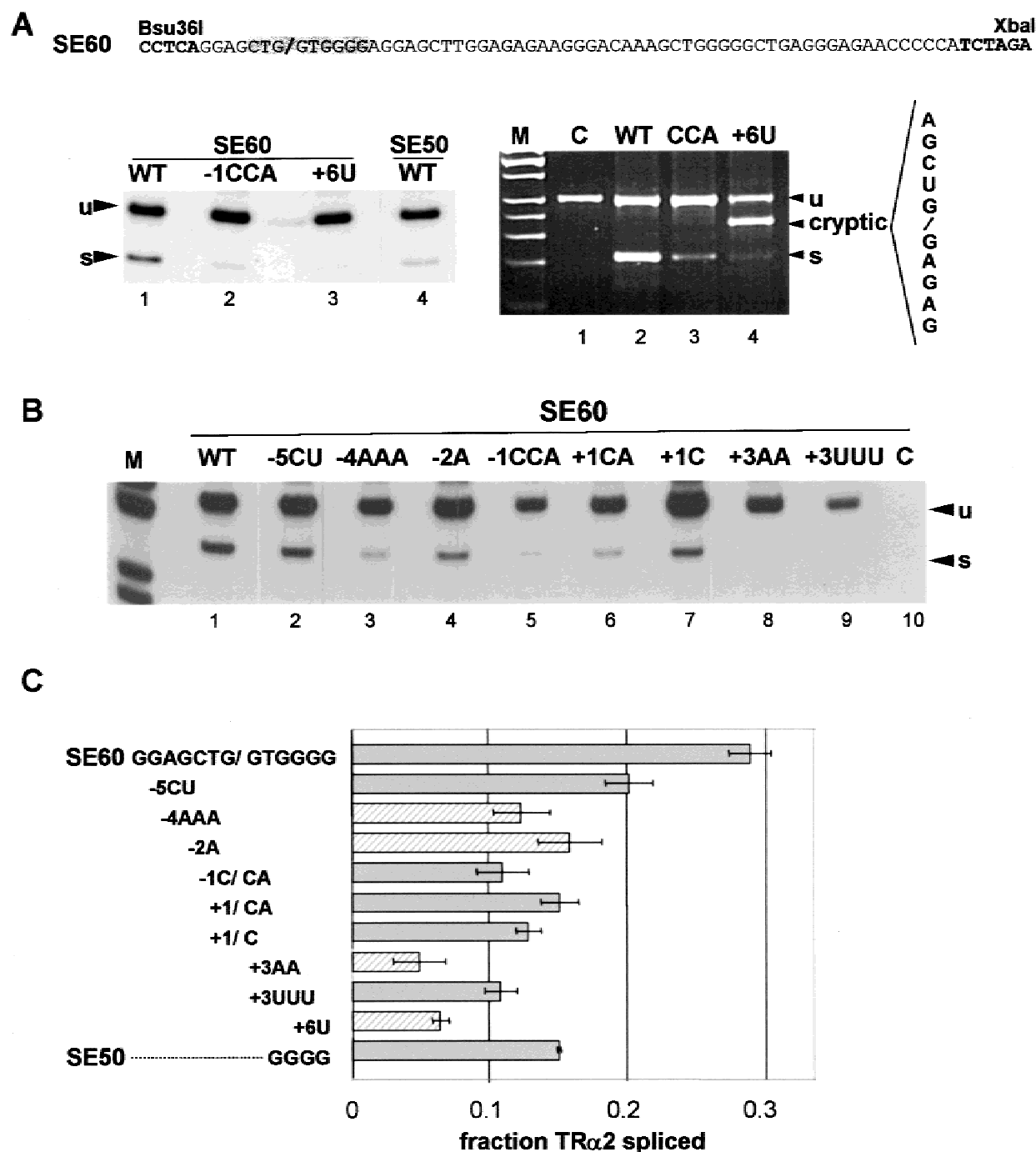


FIGURE 8. Mutations in SE60 alter TR α 2 splicing. **A**, top: Sequence of enhancer region in mutated TR α 2 substrates. Location of the 5'-splice-site-like sequence is shaded. Slash (/) indicates putative cryptic 5' splice site cleavage. Left panel: RNase protection assays carried out as described in Figure 7B with indicated mutations. Unspliced (u) and spliced (s) products are indicated. Right panel: RT-PCR analysis of total RNA isolated from wild-type and mutant SE60. A control PCR reaction (C) provides a marker for unspliced product (u). The sequence of the middle RT-PCR product band indicating cryptic splicing (/) of SE60(+6U) is shown at right. **B**: RNase protection assays carried out with SE60 RNA mutations. **C**: Histogram of TR α 2 splicing of wild-type and mutant SE60 RNA. Solid bars indicate the sole use of the TR α 2 5' splice site and hatched bars indicate RNAs that are also spliced at the cryptic 5' splice site. Results are based on two experiments and three determinations, except for SE40, which is only one determination.

GTRAGT). Additional mutations were made to determine whether this 5'-splice-site-like sequence is functionally important. Three nucleotides, GGT, which represent the most highly conserved portion of the 5' splice site consensus sequence, were replaced with CCA (SE60-1CCA; -1 indicating the position of the 5' nucleotide relative to its position in the 5' splice site consensus sequence; cf. Fig. 8A) to weaken any possible interactions with splicing factors. Conversely, the final G residue was replaced with a T to improve its match to the 5' splice site consensus sequence (SE60+6U) (Fig. 8A). If this sequence functions by binding splicing factors that promote TR α 2-specific splicing, the SE60-1CCA mutation should disrupt enhancer activity. On the other hand, if the 5'-splice-site-like sequence interacts directly with active splicing factors, the +6U mutation might either increase splicing at the normal site or promote cryptic splicing by creation of a strong 5' splice site.

The effects of these two mutations, as analyzed by RNase protection assays, indicate that both alterations cause a strong decrease in splicing at the TR α 2-specific 5' splice site (Fig. 8A, left panel, lanes 1-3) to 25-30% of that observed for the wild-type SE60. RT-PCR (Fig. 8A, right panel, lanes 2 and 3) was used to assay for normal and cryptic splicing of the transcripts. Although the -1CCA mutation showed only the expected decrease in normal splicing, RT-PCR amplification of SE60+6U RNA showed the presence of a novel band that was intermediate in length between the TR α 2 unspliced and spliced products (Fig. 8A, right panel, lane 4). Sequencing of the isolated product confirmed that the cDNA represents a novel transcript in which the 5'-splice-site-like sequence within SE60 is spliced precisely to the TR α 2-specific 3' splice site (Fig. 8A). Taken together, these results strongly suggest that the 5'-splice-site-like sequence (subsequently referred to as the pseudo-5' splice site) is important for efficient TR α 2 splicing. Interestingly, the level of TR α 2-specific splicing observed with the -1CCA mutation was consistently less than that seen with SE50 (Fig. 8A, lane 4).

To further investigate the role of this pseudo-5' splice site element, additional mutations were made containing replacements of 1 to 3 nt. Several mutations were targeted at positions corresponding to the most highly conserved nucleotides in the consensus 5' splice site. Single and double nucleotide substitutions at positions +1 and +2 of the pseudo-5' splice site (+1CA and +1C) targeted the highly conserved GT dinucleotide. Each of these was nearly as effective as the 3-nt substitution -1CCA (Fig. 8C). When the three consecutive G residues at positions +3 to +5 within the pseudo-5' splice site consensus sequence were replaced with U residues (+3UUU), splicing activity also was reduced nearly to the same level as with the -1CCA (Fig. 8B, lanes 5 and 9). Furthermore, increasing the match to

the 5' splice site by replacing G residues at positions +3 and +4 with A residues (+3AA; Fig. 8B, lane 8) strongly activated cryptic splicing (Fig. 8B, lane 8, and results not shown). These results demonstrate that the sequences included within the pseudo-5' splice site are important for TR α 2-specific splicing.

To determine if nucleotides immediately upstream of the pseudo-5' splice site in SE60 are also essential for TR α 2 splicing, two additional substitutions were examined. One substitution, -4AAA, that overlaps the -3 and -2 positions of the pseudo-5' splice site reduced normal splicing (Fig. 8B, lane 3) but activated cryptic splicing (result not shown). Further analysis showed that replacement of only a single nucleotide in this region (-2A) was sufficient for activation of cryptic splicing (Fig. 8B, lane 4, and result not shown). However, another upstream substitution, -5CU, that did not overlap any part of the 5' splice site consensus sequence, resulted in only a modest (30%) decrease in splicing relative to the substitutions studied in the pseudo-5' splice site itself (Fig. 8B, lane 2, Fig. 8C). This mutation replaces the AG dinucleotide in the center of the sequence (-7)GGAGCTG that resembles a motif (GGGGCTG) important for the activity of other intronic splicing enhancers (Carlo et al., 1996; Carstens et al., 1998). Thus, it appears that the pseudo-5' splice site is more important than the GGAGCTG element in SE α 2. Based on these results, we conclude that the pseudo-5' splice site at the 5' end of SE60 (Fig. 8A) is critically important for the splicing enhancer activity of SE α 2.

Mutations in SE α 2 that block splicing also decrease protein crosslinking

The enhancer sequences corresponding to the 5' deletions described above were compared to SE80 for their ability to crosslink proteins in HeLa cell nuclear extract. As shown in Figure 9A, lanes 1 and 2, SE60 and SE80 crosslink a similar complement of proteins with the exception of smaller proteins below 22 kDa. In contrast, both the 55- and 34-kDa proteins crosslink to SE50, SE40, and SE60-1CCA to a much lesser extent (Fig. 9A, lanes 3-5). Significantly, the SE60+6U mutation does not affect the crosslinking efficiency to the 34- and 55-kDa proteins (Fig. 9A, lanes 2 and 6). Thus, the presence of a functional 5' splice site in this fragment does not alter its interaction with these proteins.

To confirm that hnRNP H is specifically bound to SE60 but not SE60-1CCA, splicing reactions containing nuclear extract and labeled RNA were crosslinked, and bound proteins were immunoprecipitated with an hnRNP H-specific antibody. As seen in Figure 9B, hnRNP H crosslinked more efficiently to SE60 than to SE60-1CCA (lanes 3 and 7). A polyclonal antibody specific for hnRNP F also precipitated a small amount of hnRNP F, migrating slightly ahead of hnRNP

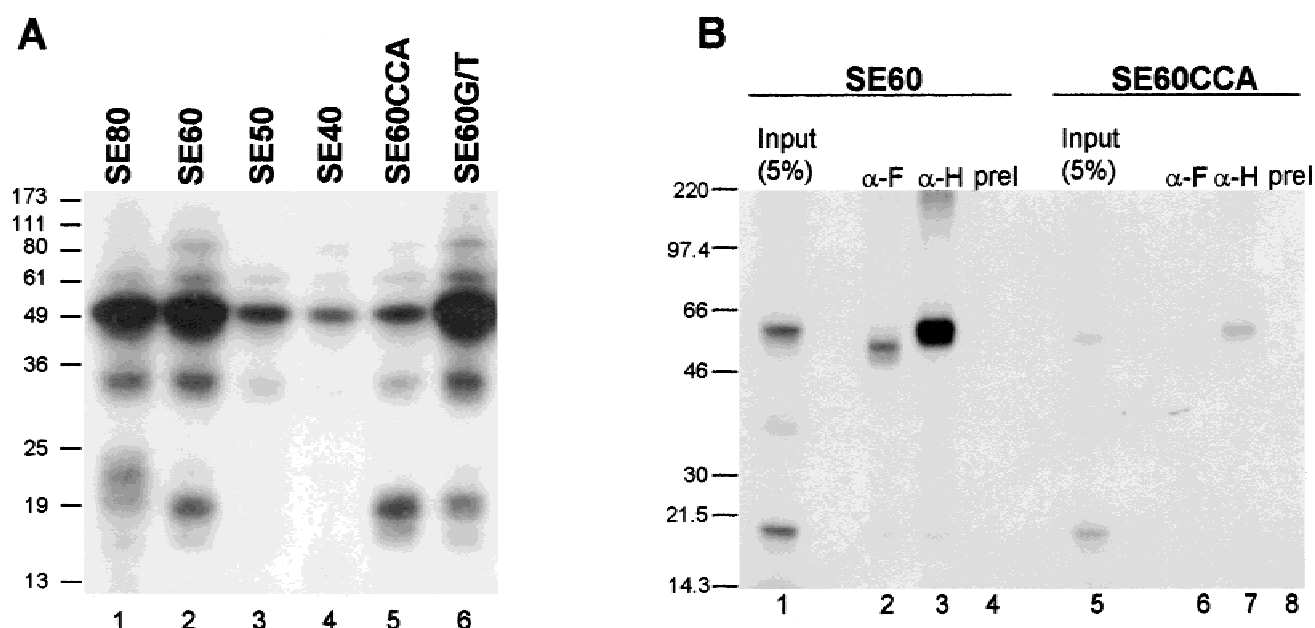


FIGURE 9. SE α 2 mutations alter protein crosslinking. **A:** Crosslinking of proteins to indicated 32 P-labeled RNAs was carried out as in Figure 5. **B:** Immunoprecipitation of crosslinked proteins with polyclonal antibodies specific for hnRNP H (lanes 3 and 7) and hnRNP F (lanes 2 and 6). Nuclear proteins were crosslinked to labeled SE60 (lanes 1–4) or SE60–1CCA (lanes 5–8) RNA with no flanking sequences followed by immunoprecipitation. Proteins were resolved by SDS-PAGE on a 10% gel.

H, which crosslinked specifically to the wild-type SE60 (Fig. 9B, lanes 2 and 6). These results show that deletions and mutations that strongly reduce the activity of the splicing enhancer also significantly reduce the crosslinking of proteins binding specifically to the purine-rich element.

DISCUSSION

Regulation of TR α 2 RNA splicing is important for determining the balance between TR α 1 and TR α 2, two functionally antagonistic transcription factors encoded by the *erbA α* gene. In this study, we show that a *cis*-acting element, SE α 2, located downstream of the TR α 2-specific 5' splice site, enhances splicing both in vivo and in vitro. This element contains several purine-rich elements similar to those found in many splicing enhancers as well as a pseudo-5' splice site. SE α 2 is different from most purine-rich enhancer elements in its intronic location (Hastings et al., 2000). We also find that SF2/ASF, hnRNP H, and hnRNP F interact with SE α 2 and that mutations in SE α 2 that weaken these interactions strongly reduce the enhancer activity of SE α 2.

Purine-rich elements are required for SE α 2 activity

SE α 2 is extremely purine rich and contains multiple copies of two motifs that have been described in other splicing enhancers. First, three uninterrupted stretches of purine residues (underlined in Fig. 1A), that include

repeats of the trinucleotide GAR, are important for splicing. When these GAR repeats were altered by eight purine–purine substitutions, a 50% decrease in splicing activity was observed. This result is consistent with other studies showing that tandem repeats of GAR are essential for enhancer activity (Xu et al., 1993; Tanaka et al., 1994; Humphrey et al., 1995).

A second type of purine-rich element in SE α 2 consists of several consecutive G residues (overlined in Fig. 1A). Such G tracts have been found within introns close to intron/exon junctions (Nussinov, 1988; McCullough & Berget, 1997, 2000). Consecutive G residues are found in certain sequence motifs present in other intronic enhancers including two, GGGGCUG and (A/U)GGG (Sirand-Pugnet et al., 1995; Carlo et al., 1996; Carstens et al., 1998) that are also present in SE α 2. Because four of the five G tracts within SE α 2 are disrupted in SE80G/U, which lacks enhancer activity, these elements may also be essential for SE α 2 function. Further deletion and substitution mutations demonstrate that sequences other than the GAR repeats are important for enhancer function. However, the G/A substitutions disrupt the GAR repeats while extending, not disrupting, two overlapping runs of three consecutive G residues in SE α 2. Thus, the GAR repeats themselves may be needed for full enhancer function.

The location of SE α 2 within an intron and its function in promoting upstream 5' splice site activity are unusual for a purine-rich enhancer, the majority of which are located within exons downstream of the 3' splice site on which they act (reviewed in Hertel et al., 1997).

Both the sensitivity of SE α 2 to the G/A substitutions and its ability to enhance splicing of the heterologous dsx pre-mRNA are consistent with characteristics previously noted for purine-rich exonic enhancers (Humphrey et al., 1995). The juxtapositioning of elements characteristic of both exonic and intronic splicing enhancers in SE α 2 is consistent with the growing body of evidence that splicing enhancers are composite elements comprised of multiple sites for binding a variety of *trans*-acting factors (Hertel et al., 1997).

SR proteins interact with SE α 2

Inhibition of TR α 2 splicing by excess wild-type competitor but not mutant transcripts suggests that SE α 2 function requires sequence-specific binding of *trans*-acting factors in HeLa nuclear extract. RNA-protein crosslinking reveals that 55-kDa, 34-kDa, and 22-kDa proteins bind to SE α 2 (Fig. 5A). Both the 22-kDa and 34-kDa proteins appear to be SR proteins (Fig. 5B). There is a single well-characterized SR protein of 20 kDa (SRp20) and at least four SR proteins that migrate at about 34 kDa (SF2/ASF, SC35, 9G8, or SRp30c; reviewed in Graveley, 2000). SF2/ASF is at least one of the proteins interacting with SE α 2, as indicated by UV crosslinking, immunoprecipitation, and northwestern blot analysis (Figs. 5B and 6A). Binding of the 22-kDa protein is competed by both the wild-type and SE80G/U competitor RNA (Fig. 5A). Although this result may indicate nonspecific binding, the protein also crosslinks much less efficiently to SE60 than to SE80 (Fig. 9A), suggesting that the 22 kDa protein may be associated with the 5' end of SE80, which is absent in SE60 and unchanged in SE80G/U.

SR proteins have been shown to mediate the activity of other purine-rich exonic splicing enhancers (reviewed in Graveley, 2000). Consistent with this role in splicing, enhancer sequences have been shown to contain consensus sequences recognized by a number of SR proteins (Liu et al., 1998; Tacke & Manley, 1999). SE80 contains an unusually high density of potential SF2/ASF interacting sites (not shown) as determined by sequence analysis with a score matrix generated by selection for SF2/ASF-dependent enhancer sequences *in vitro* (Liu et al., 1998). SE α 2 also includes two GGACAA sequences that were identified as SF2/ASF-dependent enhancers in β -globin (Schaal & Maniatis, 1999). This may explain the inhibition of β -globin splicing in the presence of SE α 2 competitor RNA (Fig. 4). In addition, both the SE80G/A and SE80G/U mutations alter potential SF2/ASF interactions. Thus, it is likely that SF2/ASF plays a role in mediating enhancer activity of SE α 2.

The requirement for SR proteins in mediating the enhancer activity of SE α 2 was further investigated by adding recombinant SF2/ASF to splicing reactions preincubated with SE80 competitor RNA. Increasing amounts of SF2/ASF relieved the competition (not

shown). This result supports the model in which SF2/ASF promotes splicing through its association with the SE α 2 enhancer. However, because SF2/ASF added to control reactions without competitor also stimulated splicing of the SE80 transcript, it is not possible to resolve completely the roles of SF2/ASF in basal splicing and in SE α 2 enhancer-mediated splicing.

hnRNP H and hnRNP F interact with SE α 2

Like the 55-kDa crosslinked protein, both hnRNP F and hnRNP H proteins are present in S100 and nuclear extract but not in the SR protein preparation, migrate between 50 and 60 kDa, and bind G-rich intronic splicing enhancer elements (Matunis et al., 1994; Chou et al., 1999). We confirmed this interaction by antibody-specific immunoprecipitation of hnRNP H and F crosslinked to SE80. The intense 55-kDa band corresponds to hnRNP H whereas hnRNP F comigrates with a much fainter band just in front of hnRNP H (Figs. 6C and 9B). Although this result may simply reflect differences in the efficiency of crosslinking and immunoprecipitation, it is likely that hnRNP H is the major protein species bound to SE α 2 and that hnRNP F binds to only a relatively small fraction of SE α 2 transcripts. SE60, which displays a high level of splicing enhancer activity (Fig. 7B), also binds hnRNP H and F (Fig. 9B), whereas SE60-1CCA, SE50, and SE40, which lack significant splicing enhancer activity, do not bind efficiently to hnRNP H. Thus, interactions of SE α 2 with hnRNP H and F correlate with splicing enhancer activity, suggesting that these proteins are involved in SE α 2 function.

hnRNP H and hnRNP F have been shown to activate neural-specific splicing of c-src mRNA (Min et al., 1995; Chou et al., 1999). This enhancer sequence is located in the intron downstream of the regulated exon. The Rous sarcoma virus (RSV) primary RNA transcript also contains an intronic element, NRS, that interacts with hnRNP H to regulate splicing (Fogel & McNally, 2000). However, in this case, hnRNP H mediates NRS-dependent splicing inhibition. hnRNP H is also implicated in the inhibition of rat β -tropomyosin splicing (Chen et al., 1999). As observed with the c-src enhancer, hnRNP H bound to SE α 2 may promote TR α 2 splicing. Inhibition or enhancement of splicing by hnRNP H does not appear to be determined by the location of the regulatory sequences but is likely to be influenced by specific interactions with other factors.

A pseudo-5' splice site sequence is critical for SE α 2 activity

Deletion analysis of SE α 2 showed that the 9-nt region present in SE60 but not SE50 overlaps a 5'-splice-site-like sequence that is essential for SE α 2 activity (Fig. 7). Interestingly, two single nucleotide substitutions (-2A

or +6U) at relatively peripheral sites, as well as other substitutions that increase the match to the consensus 5' splice site (SE60-4AAA, SE60+2AA), activate efficient cryptic splicing at this site. This cryptic splicing does not occur in wild-type pre-mRNAs and thus the sequence represents a pseudo-5' splice site.

The importance of this site in SE α 2 enhancer activity was demonstrated by additional mutations. SE60+1C RNA, which has a single base substitution in the pseudo-5' splice site sequence at one of the most highly conserved positions in the consensus 5' splice site, severely blocks splicing at the TR α 2 5' splice site (Fig. 8). However, it does not appear that this pseudo-5' splice site is entirely responsible for SE α 2 activity, as other substitutions (SE80A/G, SE60-5CU) and deletions (SE60 and SE60/3') within SE α 2 that do not affect this sequence also reduce TR α 2 mRNA splicing.

Pseudo-5' splice sites have been identified within elements regulating alternative processing in other transcripts. Pseudo-5' splice sites located in the drosophila P element (Siebel et al., 1992) and Rous sarcoma virus (Gontarek et al., 1993) mediate inhibition of alternative splicing, whereas one in calcitonin/CGRP mRNA activates alternative polyadenylation at an upstream site (Lou et al., 1995). Each of these sites has been shown to interact with U1 snRNP (Siebel et al., 1992; Lou et al., 1998; Hibbert et al., 1999; McNally & McNally, 1999). However, in no other instance is a pseudo-5' splice site sequence associated with the activation of splicing as it is in SE α 2. Recently, intronic G triplets present in α -globin pre-mRNA have been shown to bind U1 snRNPs and enhance splicing at an upstream 5' splice site, indicating that U1 snRNP binding within an intron can stimulate splicing (McCullough & Berget, 2000). The role of the SE α 2 pseudo-5' splice site in activating splicing of TR α 2 pre-mRNA is reminiscent of the ability of the 5' splice site of a downstream intron to stimulate splicing of an upstream intron, an activity ascribed to exon definition (Robberson et al., 1990). However, because the intronic pseudo-5' splice site sequence appears to promote the use of an upstream 5' splice site rather than an upstream 3' splice site, as in traditional exon definition, the mechanism by which it promotes splicing is likely distinct. Thus, the pseudo-5' splice site present in SE α 2 provides an unusual example of the ability of 5'-splice-site-like sequences to activate splicing at a distance.

Models for SE α 2 splicing enhancer activity

One model for SE α 2 function is that factors interacting with a pseudo-5' splice site promote the binding of other hnRNP and SR proteins which, in turn, promote use of the TR α 2-specific 5' splice site upstream. The SE60-1CCA mutation may abrogate associations with factors such as U1 snRNP binding to the pseudo-5'

splice site, and thereby weaken interactions of proteins such as SF2/ASF and hnRNP proteins with neighboring sites in the enhancer. In this model, the pseudo-5' splice site serves as a recruitment or assembly site for components of an enhancer-associated complex. Alternatively, SR proteins, hnRNP H and F, and other factors may assemble at or near the pseudo-5' splice site sequences independent of U1 snRNP binding and stimulate assembly of other splicing factors including U1 snRNP, which, although not active itself, is properly situated to promote processing at the upstream TR α 2-specific 5' splice site. Indeed, SR proteins have been reported to stimulate U1 snRNP binding at a 5' splice site (Kohtz et al., 1994; Staknis & Reed, 1994; Jamison et al., 1995). These models are supported by our results that both the pseudo-5' splice site and purine-rich region are functionally important, as neither element is efficient in enhancing TR α 2 splicing in the absence of the other (Figs. 1 and 8). Such models for SE α 2 splicing enhancer activity are attractive, as the balance between TR α 2 splicing and TR α 1 polyadenylation is dependent on a number of factors, including the relative strength of the TR α 2-specific splice sites, the TR α 1 polyadenylation site, and SE α 2 (Hastings et al., 2000; S.H. Munroe, M.L. Hastings, & S. Balthazor, unpubl. results).

The results presented here define an unusual intronic enhancer element that is important for the alternative processing of mRNAs for two major isoforms of the mammalian T3 receptor. We have identified three components, SF2/ASF, hnRNP F, and hnRNP H, that interact specifically with SE α 2. Like other splicing enhancer elements, SE α 2 appears to establish a balance between competing processing reactions. However, several lines of evidence suggest that regulation of the levels of TR α 1 and TR α 2 mRNA also involves selective inhibition of TR α 2 mRNA processing by a naturally occurring RevErbA α antisense transcript that overlaps the 3' end of TR α 2 but not TR α 1 mRNA (Lazar et al., 1988; Hastings et al., 2000). Further studies of SE α 2 will provide insight into its role in the regulation of TR α gene expression and the function of this intronic, purine-rich splicing enhancer and its associated pseudo-5' splice site.

MATERIALS AND METHODS

Plasmids

Construction of pErbA, pErbA(Δ BS), and pErbA(SE80) has been described previously (Hastings et al., 2000). pErbA(SE80)G/U, pErbA(SE80)G/A, pErbA(SE80) Δ 30, pErbA(SE70), pErbA(SE60), pErbA(SE50), pErbA(SE40), pErbA(SE60/3'), pErbA(SE60)CCA, and pErbA(SE60)G/T were constructed from pErbA(SE80) or pErbA(SE60) by PCR using primer sets containing the desired mutations in addition to the *Bsu*36I and *Xba*I restriction sites flanking

the 80-nt enhancer. The sequences of the specific inserts in these constructs are shown in Figures 1A, 7A, and 8A. pErbA(SE80) Δ HB and pErbA(SE80)G/U Δ HB were constructed by cutting pErbA(SE80) and pErbA(SE80)G/U, respectively, with *HindIII* and *Bsu36I*, and religating the vectors after end filling to delete the 5' end of the minigene insert. Plasmids used for production of pre-mRNA in vitro splicing substrates were derived from plasmid α 2-S (Munroe & Lazar, 1991). This plasmid contains the same deletion as pErbA(Δ XS) (Hastings et al., 2000) but lacks intron 8 and sequences upstream of the *PstI* site in exon 8 and downstream of the *EcoRI* site in exon 10. α 2(Δ BS) contains the same deletion as described for pErbA(Δ BS) (Hastings et al., 2000). α 2(SE80) and α 2(SE80)G/U were constructed by inserting the sequences found between the *Bsu36I* and *XbaI* sites in pErbA(SE80) and pErbA(SE80)G/U, respectively, between the same sites in α 2(Δ BS). Plasmids pDsx-ASLV (Staknis & Reed, 1994) and pDsx modified by insertion of *XbaI* and *KpnI* cloning sites in two different orientations (McNally & McNally, 1998) have been previously described. The latter two pDsx vectors are referred to as pDsx-X/K and pDsx-K/X, depending on the order of the *XbaI* (X) and *KpnI* (K) sites in the polylinker region adjacent to *dsx* exon 4. The *dsx* plasmids were the generous gift of R. Reed (Harvard Medical School, Boston, Massachusetts) and M.T. McNally (Medical College of Wisconsin, Milwaukee, Wisconsin). Following trimming with *KpnI* and *XbaI*, the insert fragments were cloned into pDsx-K/X, to produce chimeric plasmids with TR α sequences cloned downstream of *dsx* exon 4. Four plasmids were constructed. pDsx-SE80, pDsx-SE80G/A, and pDsx-SE80G/U include the SE α 2 inserts from pErbA(SE80), pErbA(SE80)G/A and pErbA(SE80)G/U, respectively. pDsx-80SE80 contains the SE80 extended by an additional 80 nt upstream of the *Bsu36I* site for a total insert length of 160 nt. These inserts were generated by PCR using sense primers containing a *KpnI* site at the 5' end and antisense primers located downstream of the *XbaI* site in the corresponding pErbA derived constructs. The fragments used to construct pDsx-SE80 and pDsx-SE80G/U were also cloned into pDsx-X/K in the reverse orientation to create pDsx-SE80(R) and pDsx-SE80G/U(R), respectively. Sequences of all cloned PCR-generated inserts were confirmed by DNA sequencing.

Cell culture and in vivo RNA analysis

COS-1 and HEK 293 cells were maintained as previously described (Hastings et al., 1997, 2000). Twenty-four hours prior to transfection, logarithmically growing COS-1 or HEK 293 cells were replated at a density of 5×10^5 or 1.5×10^6 cells per 90-mm plate. Cells were transfected with 5 μ g of plasmid DNA per plate using the calcium phosphate procedure and total RNA was harvested 48 h posttransfection by Nonidet P-40 lysis (Sambrook et al., 1989). RNase protection assays were carried out and quantitated using probes and procedures previously described (Hastings et al., 1997, 2000).

In vitro splicing

All α 2 plasmids were linearized with *EcoRI* and transcribed with SP6 RNA polymerase to generate RNA substrates, as described previously (Munroe & Lazar, 1991). pHB500 was

cut with *BamHI* and transcribed with T7 RNA polymerase to obtain the globin transcript as previously described (Munroe, 1988). The chimeric pDsx-SE80 plasmids were linearized with *XbaI* and transcribed with T7 RNA polymerase. pDsx-X/K and pDsx-ASLV were cut with *XbaI* and *MluI*, respectively, and transcribed with T7 RNA polymerase. In vitro splicing assays were performed with HeLa nuclear extract as previously described using the specific conditions described for splicing β -globin, TR α , and *dsx* transcripts (Munroe, 1988; Munroe & Lazar, 1991; Tian & Maniatis, 1993). Competitor RNAs SE80 and SE80G/U were made by transcribing pDsx-SE80(R) and pDsx-SE80G/U(R), respectively, with SP6 RNA polymerase after linearizing with *XbaI*. These transcripts were 133 nt in length, including the 80-nt SE80 sequence and 53 nt of flanking polylinker sequence. Another set of SE80 and SE80G/U competitor RNAs was made by transcribing pErbA(SE80) Δ HB and pErbA(SE80)G/U Δ HB with T7 RNA polymerase after linearizing with *XbaI*. These transcripts contained only 16 nt of a different flanking sequence. Nonspecific competitor RNA was transcribed from pDsx linearized with *HaeIII* and transcribed with SP6 to generate a 130-nt RNA transcript containing 85 nt of antisense *dsx* sequence and additional polylinker sequence identical to that in the SE α 2 competitor RNAs. Competitor RNAs were uniformly labeled with 3 H-CTP to facilitate quantitation based on the specific base composition of each transcript. The cap analog m 7 GpppG was incorporated into the 5' end of each splicing substrate, but omitted from the competitor RNAs. Competition experiments were performed by preincubating competitor RNAs with HeLa nuclear extracts for 10 min at 30 °C prior to adding the 32 P-labeled substrate. RNA products were extracted, precipitated, subjected to electrophoresis in polyacrylamide/urea gels, and quantitated on an AMBIS radioanalytic imaging system as previously described (Munroe, 1988; Hastings et al., 1997). The splicing efficiency was calculated as the percentage of fully spliced RNA divided by the sum of spliced products and the remaining unspliced pre-mRNA after correcting for length and base composition.

RNA substrates SE80, SE60, SE50, SE40, SE60CCA, and SE60G/T used in splicing, UV-crosslinking, and immunoprecipitation reactions in Figures 6C and 9 were prepared by PCR of pErbA(SE80) with a sense primer that includes the T7 promoter (TAATACGACTCACTATAGGG) followed by the last two G residues of the *Bsu36I* site and an antisense primer within exon 10. The PCR products were linearized with *XbaI* and transcribed with T7 RNA polymerase to give RNAs with no flanking sequences. The crosslinking pattern of SE80 was identical to that of SE80 transcripts from plasmids linearized with *XbaI*.

RNA-protein crosslinking

Ten nanomoles 32 P-labeled SE80 RNA transcribed from pErbA(SE80) Δ HB were incubated under splicing conditions (without polyvinyl alcohol) in the presence or absence of a 1.25- to 5-fold molar excess competitor RNA with HeLa cell nuclear extract, S100 fraction, or an SR protein fraction (Krainer et al., 1984; Krainer & Maniatis, 1985; Zahler, 1999). After 30 min at 30 °C, the reactions were put on ice and exposed to 254 nm UV light using a Spectronics XL-1000 UV crosslinker at a setting of 18 mJ/cm 2 . Samples were digested with RNase A (10 μ g) and RNase T1 (100 U) for

15 min at 37 °C. Crosslinked proteins were analyzed by SDS polyacrylamide gel electrophoresis, followed by autoradiography. Sets of SE80 competitors, with long, short, or no flanking regions, were used with identical results in UV-crosslinking experiments.

Immunoprecipitation of crosslinked proteins

For immunoprecipitation of SF2/ASF and hnRNP H and F bound and UV crosslinked to RNA, protein A-Sepharose beads (15 μ L; Pharmacia) were bound overnight at 4 °C to either an SF2/ASF specific antibody, mAb96, or to a maltose-binding protein-specific antibody, mAb105 control antibody (Hanamura et al., 1998). Antibodies specific for hnRNP H (a gift of D. Black, University of California, Los Angeles/Howard Hughes Medical Institute; Chou et al., 1999), hnRNP F, and pre-immune serum (a gift from C. Milcarek, University of Pittsburgh) were bound to protein A-Sepharose beads for 3 h at 4 °C. The beads were washed three times with immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% NP-40). UV-crosslinking reactions, performed as described above except crosslinked at 1.8 J/cm², were added to the antibody-bound beads and mixed for 2 h at 4 °C followed by three washes in immunoprecipitation buffer. Immunoprecipitated proteins were eluted from the beads by boiling for 5 min in SDS-PAGE loading buffer and were resolved by SDS-PAGE.

Northwestern and western blot analysis

HeLa nuclear extract, S100 extract, total HeLa SR proteins, recombinant proteins SF2/ASF, expressed in *Escherichia coli* and purified (Screaton et al., 1995), and baculovirus-expressed SC35 were run on a 12% SDS polyacrylamide gel and electroblotted onto Protran nitrocellulose membrane. Northwestern blots were blocked by incubating 1 h in 5% nonfat dry milk in 25 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM HEPES, pH 8.0. RNA binding was carried out by incubating the blot with ³²P-labeled SE α 2 RNA (10⁶ cpm) in RNA binding buffer (50 μ g/mL tRNA, 2.5% nonfat dry milk, 50 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 10 mM HEPES, pH 8.0). After a 30-min incubation at room temperature, samples were washed three times in RNA binding buffer. For western blots, membranes were blocked by overnight incubation in 5% nonfat dry milk in TBST (10 mM Tris-Cl, pH 8, 150 mM NaCl, and 0.05% Tween) and washed with TBST. Blots were probed with SF2/ASF monoclonal antibody mAb96 (1:20) for 1 h, washed with TBST, incubated with HRP-conjugated goat anti-mouse, and developed with ECL western blotting detection reagent (Amersham).

ACKNOWLEDGMENTS

We thank Heather Shoven for skillful technical assistance, Mark McNally and Robin Reed for recombinant plasmids, Adrian Krainer, Doug Black, and Christine Milcarek for antibodies and protein reagents, and Mark McNally and Mitch Lazar for helpful comments on the manuscript. This work was supported by National Institutes of Health Grants DK 48034 and GM55922 to SHM. MLH was supported by a predoctoral fellowship from the Arthur J. Schmitt Foundation.

Received October 12, 2000; returned for revision November 2, 2000; revised manuscript received March 26, 2001

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